



## Research Project Summary



### The September 2002 Version of the Method 1600 Enterococcus Test: *Aerococcus viridans* as a False-positive Organism

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#### Abstract

When analyzing marine bathing water samples using the September 2002 version of the USEPA Method 1600 enterococcus test, *Aerococcus viridans* was infrequently observed as a high-rate “false-positive” organism. The USEPA intends to revise Method 1600 to eliminate the counting of blue-halo colonies less than 0.5 mm diameter, but users of the September 2002 or earlier version of Method 1600 should be aware of this false-positive issue.

Since May 2004, to comply with the federal BEACH Act (12) and subsequent U.S. Environmental Protection Agency (USEPA) requirements (15, 18), the New Jersey Department of Environmental Protection (NJDEP) has determined the sanitary quality of NJ marine bathing beach waters using the September 2002 version of the USEPA Method 1600 enterococcus test (10,16).

Method 1600 is a test in which a sample of water is filtered and the filter rolled onto an enterococcus-selective growth medium and incubated for 24 h at 41°C. In the September 2002 version of the test, all bacterial colonies with a blue halo, regardless of colony size, are counted as enterococcus (16). The appearance of the blue halo is due to the presence of a ( $\beta$ -glucosidase enzyme that cleaves indican (indoxyl-( $\beta$ -D-glucoside), a colorless compound, which recombines to form blue indigo. The test method states that there is a 6 % false-positive rate (and a 6.5% false-negative rate, 16). It is assumed that this is an average false-positive rate (a range is not provided). Higher false-positive rates have been observed by other investigators (2, 21).

In NJ, 325 ocean and bay bathing beach locations are sampled weekly and tested by the coastal county (as well as a few municipal, regional, and township) health departments for the presence of enterococcus organisms using Method 1600. The concentration of enterococcus in these samples may not exceed 104 per 100 ml (the upper 75th percentile confidence interval of the acceptable geometric mean concentration for marine waters 18). Exceeding this value requires immediate re-sampling and a sanitary survey of the area. Two consecutive violations result in closure of the beach to primary contact recreational

activities. Daily monitoring is continued until an acceptable enterococcus value and sanitary survey result is obtained and the beach is then re-opened.

Enterococcus concentrations in samples from several ocean and bay bathing beaches were unusually high (> 1000 per 100 ml), often in the absence of high concentrations at adjacent or nearby beaches. At one of these locations, simultaneous testing of the sample for enterococcus, fecal coliform (1) and *E. coli* bacteria (17) revealed counts of 1400 (“1400-Count”), <3, and <3 per 100 ml respectively. A previous analysis of 87 NJ marine (not surf-zone) waters between 1990 and 1994 for enterococcus and fecal coliform bacteria revealed a high correlation ( $r = 0.88$ ) of the respective concentrations. Thus, the absence of detectable coliform bacteria in the 1400-Count enterococcus sample was surprising.

Ten well-isolated colonies from the 1400-Count sample plate were randomly selected, identified as gram positive cocci and subjected to enterococcus confirmatory testing as specified in Section 15 of the method (16). None of the colonies verified as *Enterococcus* spp. All colonies from the 1400-Count plate (Fig. 1A) appeared near the end of the 24 h incubation period, were less than 0.5 mm in diameter, and created lighter-blue halos than colonies that verify as *Enterococcus* spp. (Fig. 1B).

Nine colonies <0.5 mm dia (excluding halo) were randomly selected from high-concentration sample plates from 4 bathing beach sites from two counties and subjected to identification using the “API 20 Strep” gram positive bacterial identification system (bioMerieux Inc, 100 Rodolphe St, Durham, NC 27712). Six colonies were identified as *Aerococcus viridans* and three could not be classified. Additional identification procedures such as 16S ribosomal DNA analyses (e.g., 7) were not attempted.

An environmental sample showing both *Aerococcus viridans* and *Enterococcus* spp. colonies is shown in Figure 1C.

In early July 2004 the NJDEP requested guidance from the USEPA regarding the counting of these small-diameter colonies. The NJDEP received written guidance from the USEPA recommending that colonies less than 0.5 mm diameter no longer be counted as enterococcus, further stating their intention to revise Method 1600 to this effect by the end of 2004 (13). The NJDEP immediately instituted the revised counting procedure resulting in a reduction of some sample counts and need for closures at several beach locations. Occasional high-concentration enterococcus samples continue to be observed at some beach sampling locations, typically associated with wet weather conditions.

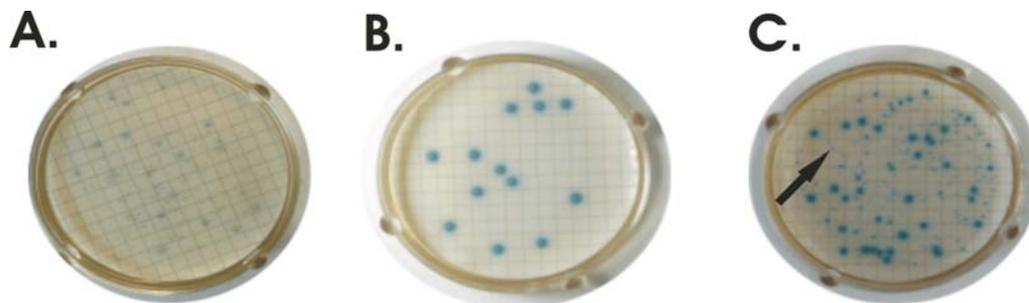
*Aerococcus viridans* and a few other non-enterococcus lactic acid bacteria (Order: *Lactobacillales*) are known to possess  $\beta$ -glucosidase enzyme (3). Interference by *A. viridans* has been observed in commercial enterococcus detection tests that rely on the presence of the  $\beta$ -glucosidase enzyme (8). Niemi and Niemela (9) showed that *A. viridans* #20340 was able to grow, albeit poorly, on Bile Esculin Azide Agar liquid medium at 41, 42 and 44 but not 45°C. Based on the late development of colonies on the 1400-Count sample plate, it is possible that the observed *A. viridans* bacteria grew at or near their upper temperature tolerance limit.

*Aerococcus viridans* was first described in 1953 (20) and has been observed in many non-fecal

environments (6, 11). It is a well-known pathogen of lobsters and other crustaceans (14) and is an occasional opportunistic pathogen in humans and animals (5, 6). Williams et al. (20) noted that aerococci are “by no means common in [human] faeces.” *A. viridans* has been found in the feces of minks “less frequently” than other gram-positive bacteria including several species each of enterococcus and streptococcus, but quantitative data was not provided (19). The authors are unaware of other reports of the occurrence of this organism in feces. Thus, the presence of *A. viridans* in marine water appears to have little sanitary significance. Due to its many similarities to *Enterococcus* spp. (4), *A. viridans* is likely under-reported in the environmental literature (20).

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**Figure 1**

- A. Method 1600 non-enterococcus colonies (< 0.5 mm) identified as *A. viridans*.
- B. Method 1600 colonies that verify as *Enterococcus* spp.
- C. Method 1600 environmental sample showing *Aerococcus viridans* (arrow pointing to < 0.5 mm dia colony) and *Enterococcus* spp. colonies.

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